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Published in:
Cell Surface

DOI:
[10.1016/j.tcs.2021.100059](https://doi.org/10.1016/j.tcs.2021.100059)

Publication date:
2021

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Document Version
Publisher's PDF, also known as Version of record

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Jones, C. Y., Engelhardt, I., Patko, D., Dupuy, L., Holden, N., & Willats, W. G. T. (2021). High-resolution 3D mapping of rhizosphere glycan patterning using molecular probes in a transparent soil system. *Cell Surface*, 7, [100059]. <https://doi.org/10.1016/j.tcs.2021.100059>

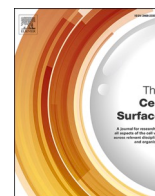
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High-resolution 3D mapping of rhizosphere glycan patterning using molecular probes in a transparent soil system

Catherine Y. Jones^{a,*}, Ilonka Engelhardt^{b,c}, Daniel Patko^{b,c}, Lionel Dupuy^{b,c,d}, Nicola Holden^c, William G.T. Willats^{a,*}

^a School of Natural and Environmental Sciences, Newcastle University, Devonshire Building, Newcastle-Upon-Tyne NE1 7RU, UK

^b Neiker, Department of Conservation of Natural Resources, Derio, Spain

^c The James Hutton Institute, Invergowrie, Dundee DD2 5DA, Scotland, UK

^d IKERBASQUE, Basque Foundation for Science, Plaza Euskadi 5, 48009 Bilbao, Spain

ARTICLE INFO

Keywords:

Rhizobacteria
Transparent Soil
Rhizosphere
Polysaccharide
Fluorescence Microscopy

ABSTRACT

Rhizospheres are microecological zones at the interface of roots and soils. Interactions between bacteria and roots are critical for maintaining plant and soil health but are difficult to study because of constraints inherent in working with underground systems. We have developed an *in-situ* rhizosphere imaging system based on transparent soils and molecular probes that can be imaged using confocal microscopy. We observed spatial patterning of polysaccharides along roots and on cells deposited into the rhizosphere and also co-localised fluorescently tagged soil bacteria. These studies provide insight into the complex glycan landscape of rhizospheres and suggest a means by which root / rhizobacteria interactions can be non-disruptively studied.

The interface of roots and soil, the rhizosphere, is a microecological zone harbouring a rich diversity of microbes (the rhizobiome) (Daniel, 2005; Wagg et al., 2014). Interactions between the root and rhizobiome are complex, dynamic and important for plant health in relation to nutrient and water uptake, defence against pathogenic and herbivorous organisms, and root colonisation by arbuscular mycorrhizal fungi (Ferreira et al., 2020). Healthy soils are characterised by having diverse rhizobiomes, and diversity is known to be strongly influenced by molecules deposited by roots into the rhizosphere (rhizodeposits) (Richardson, 2001; Mendes et al., 2013; Ofek-Lalzar et al., 2014; Tkacz and Poole, 2015; Jacoby and Kopriva, 2019). Metabolites such as hormones (e.g. strigalactone) and phenols (e.g. coumarins) have long been implicated in promoting favourable root-microbe interactions (Jacoby and Kopriva, 2019). The roles of complex polysaccharides in root-microbe interactions has received less attention, although they are abundant in the rhizobiome both as exuded mucilage, on the surface of epidermal cells and sloughed off border cells and as components of microbial biofilms. Evidence from other terrestrial and marine ecosystems demonstrates that polysaccharide complexity drives cognate microbial diversity via evolution of highly specialised carbohydrate active enzymes (CAZymes) that enable microbes to utilise glycan substrates (Flint et al., 2012; Bennke et al., 2016; Grondin et al., 2017; Lapébie et al., 2019). There is also evidence that qualitative and quantitative

variations in polysaccharide-rich root exudates and those that are surface bound have a major influence on soil microbiomes (Kuzakov and Domanski, 2000; Dennis et al., 2010; Galloway et al., 2018; Jacoby and Kopriva, 2019). In some cases, glycans have been identified that are associated with colonisation by specific microbes, for example, galactans in potato root exudates and *Pectobacterium atrosepticum* (Koronev et al., 2016). Structurally complex proteoglycans (extensins and arabinogalactan-proteins) are abundant in exudates and appear to have roles in both plant growth promotion and defence (Nguema-Ona et al., 2013; Castilleux et al., 2018). However, a detailed understanding of the roles that glycans either in rhizodeposits or on root surfaces (rhizoglycans) play in maintaining rhizobiome diversity is lacking, partly because of the technical challenges associated with sampling and analysing glycans at the precise locations where these complex root-microbe interactions occur in this underground microecological zone.

Prior work by the authors and others using polysaccharide-directed monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs) has revealed how epitope distribution varies markedly along the root surface, with characteristic and consistent glycan labelling associated with the main body of the root, the root tip, root hairs and exudates including border cells (Willats et al., 2001; McCartney et al., 2003; Guillemain et al., 2005; Wilson et al., 2015). These *in vitro* experiments show that root glycan patterning is complex and dynamic. However,

* Corresponding authors.

<https://doi.org/10.1016/j.tcs.2021.100059>

Received 2 March 2021; Received in revised form 2 July 2021; Accepted 12 July 2021

Available online 16 July 2021

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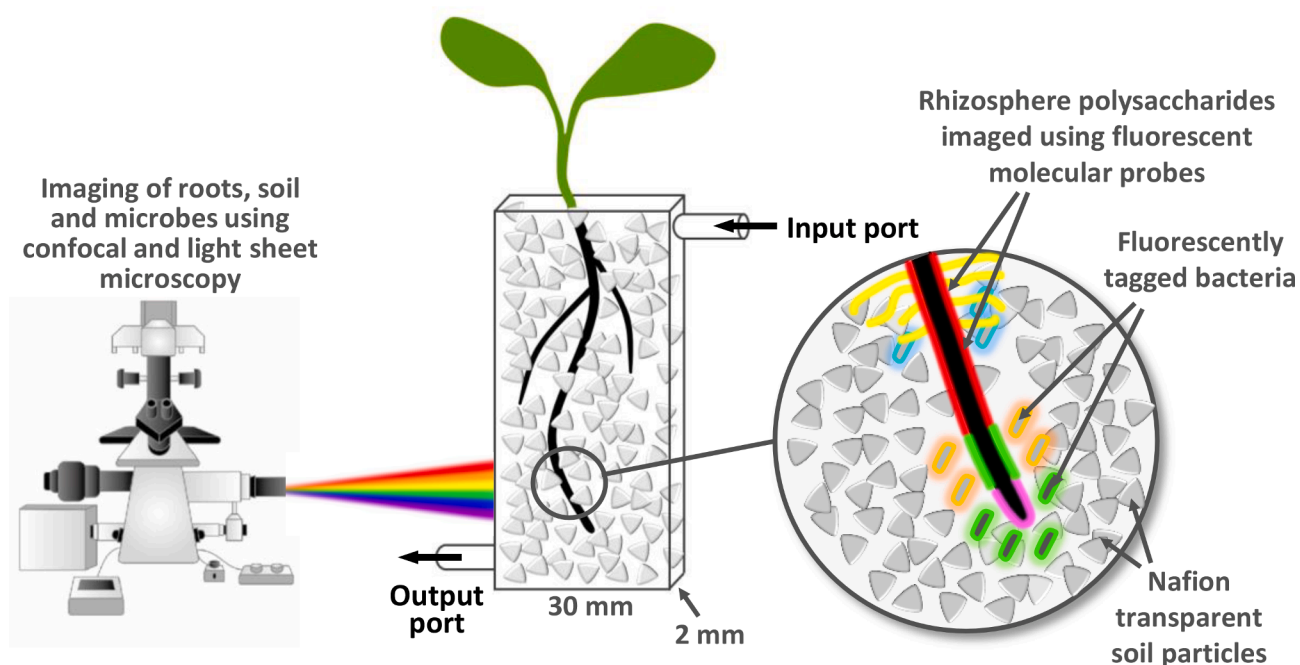


Fig. 1. A novel technology platform for an *in-situ* rhizosphere glycobiology. The transparent soil system is based on a thin glass chamber housing Nafion artificial soil particles in which seedlings grow. Bacteria, molecular probes and other components can be introduced via ports and the system washed with buffers. Using confocal and light sheet microscopy, rhizosphere polysaccharides, fluorescently tagged bacteria and Nafion particles are imaged *in-situ*.

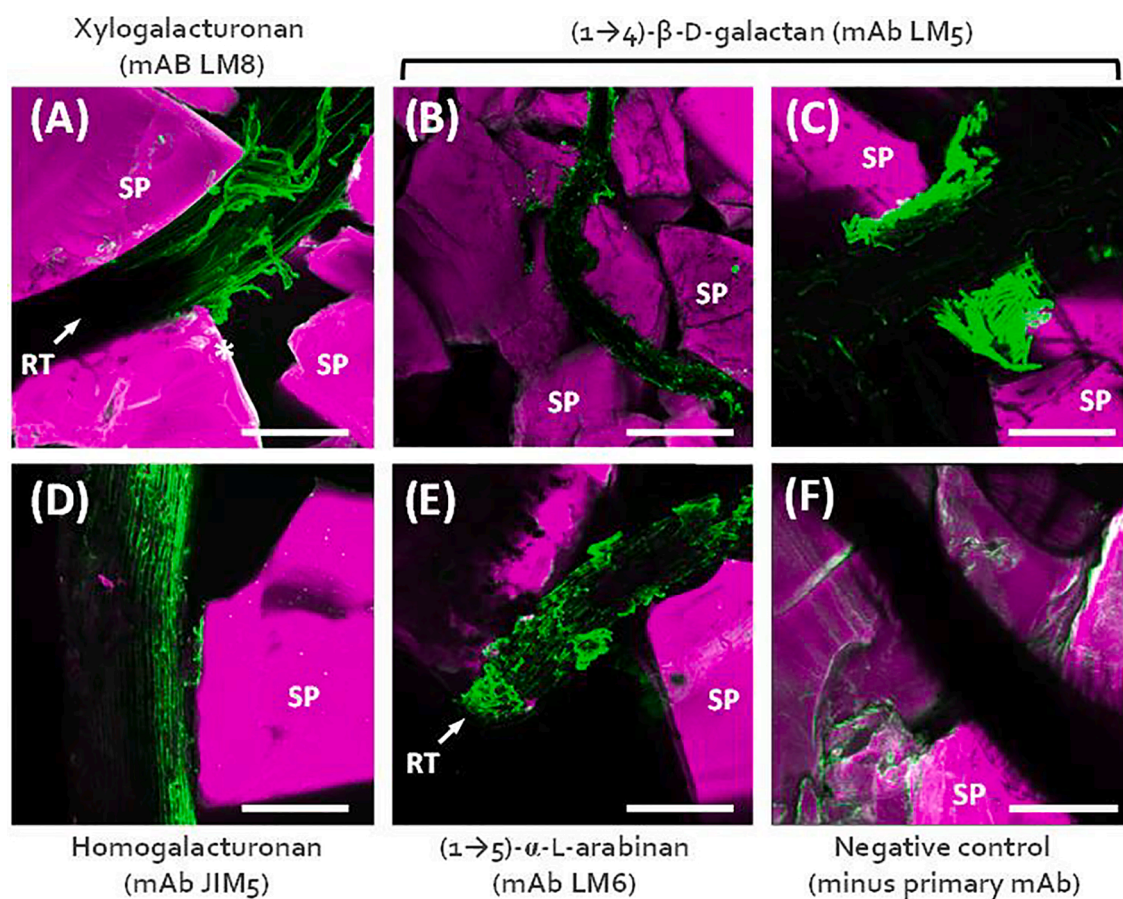


Fig. 2. Imaging root associated polysaccharides using a transparent soil system. Lettuce seedling roots growing through Nafion soil particles (SP) were labelled with mAbs with specificity for xylogalacturonan (A), (1 → 4)- β -D-galactan (B) and (C) homogalacturonan (D) and (1 → 5)- α -L-arabinan (E). RT – root tip. SP – soil particle. Scale bars = 200 μm.

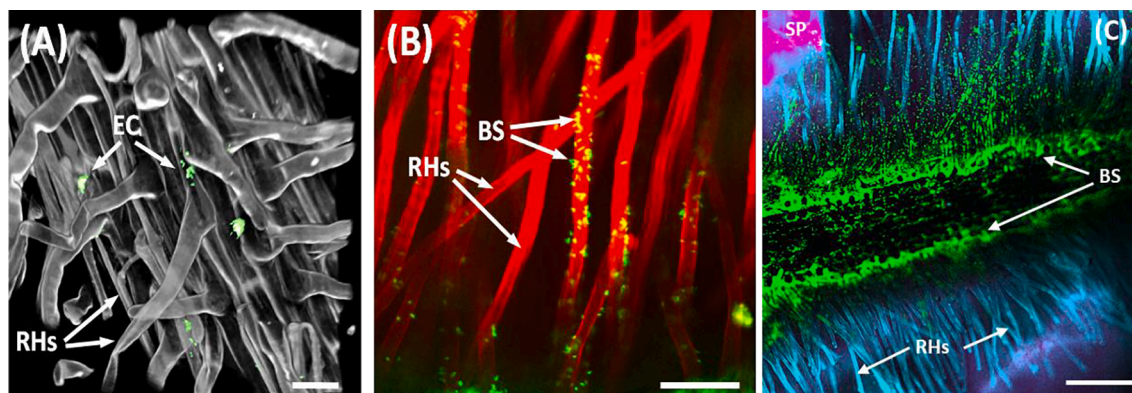


Fig. 3. *Imaging of microbes and glycans.* (A) colonies of GFP-tagged *E. coli* 0157:H7 (EC, green punctate labelling) observed within the root hair (RH) matrix of a *Lactuca sativa* root; (B) GFP-tagged *B. subtilis* (BS, green punctate labelling) were inoculated into the TSS and also colonised *Lactuca sativa* root hairs. Roots were simultaneously labelled with the anti-xyloglucan mAb LM15 (red, Alexa594) revealing that this polysaccharide was abundant on the root hair surface; (C) as in (B) showing *B. subtilis* colonies (BS, green punctate labelling) located on the main root surface with xyloglucan labelling (mAb LM15, Alexa594) displayed in blue to contrast with Naphion soil particles. Scale bars, A = 50µm, B = 100µm, C = 200µm.

they do not provide information on how this patterning changes over time with root growth and how glycan deposition may be influenced by interactions between roots and soil particles, and the effects of microbial degradation.

Specialised chambers of various kinds (rhizotrons) have been used for 125 years for observing roots in soil and for sampling material from specific locations (López-Mondéjar et al., 2016; Wilson et al., 2015; McCartney et al., 2003). More recently, X-ray computed tomography and nuclear magnetic resonance have emerged as powerful non-invasive techniques, for studying root bio physics, soil elemental composition and water distribution (Paya et al., 2015; Rogers et al., 2016; Pflugfelder et al., 2017; Yang et al., 2017; Gao et al., 2019). Nevertheless, much of the complex and dynamic biology of the rhizosphere has remained hidden. A breakthrough came with the development of transparent soil systems (TSSs). Several examples have been described and all seek to achieve a balance between reducing opacity whilst replicating nature as closely as possible (Yang et al., 2013; Ma et al., 2019; Sharma et al., 2020). The system employed for this study is based on Nafion™ particles, a tetrafluoroethylene based fluoropolymer-copolymer that is physically and chemically adaptable (Downie et al., 2012). Nafion™ particle size distribution can be manipulated by freeze milling, and minerals and fluorescent dyes can be adsorbed onto particles for controlling nutrient supply to plants, and fluorescence imaging of soil pore size and particle geometry. Plant development in this TSS is similar to that in natural soils and plants can be maintained for several weeks in this system (Downie et al., 2012; Downie et al., 2014). Here, we report the development of a new method for imaging rhizoglycan distribution and microbial colonisation that combines a Nafion™ based TSS with glycan-specific mAbs and Green Fluorescent Protein (GFP) tagged bacteria (Fig. 1). All data presented here was obtained using Leica TCS SP2 or Nikon A1R confocal laser scanning microscopes and objective lenses 10×/0.30, 20×/0.50 or 40×/0.80. Images were generated from 3D dataset using either volume rendering or projection algorithm from ImageJ or Nikon NIS-Elements software.

Germinated 5-day old lettuce seedlings were placed at the top of the TSS chamber and roots grew into the Nafion matrix. Roots were blocked using 0.5% Bovine Serum Albumin (BSA) in phosphate buffered saline (PBS) for 30 min, prior to circulation of primary mAbs (diluted to 1:10 with PBS) for 1.5 hr. Both blocking buffer and mAbs were circulated through the chamber via the upper inlet and lower outlet using a peristaltic pump. Primary mAbs were washed out by flushing the system with PBS buffer. Fluorophore tagged secondary antibodies (anti-Rat Alexa Fluor® 488, diluted to 1:100 in PBS) were used to visualise primary mAb binding. The thin and transparent TSS chamber enabled direct non-disruptive *in situ* imaging of fluorescently labelled entities

using a Laser Scanning Confocal Microscopy (LSCM), and Light Sheet Microscopy (Yang et al., 2013). Using this system, it was possible to localise specific epitopes associated with living roots and the rhizosphere in the context of soil particles. We observed localisation of extensin, xylogalacturonan, (1 → 4)-β-D-galactan, homogalacturonan and (1 → 5)-α-L-arabinan on differing tissues around the root tip zone using the primary antibodies LM1 (Smallwood et al., 1995), LM8 (Willats et al., 2004), LM5 (Jones et al., 1997), JIM5 (Knox et al., 1990; Clausen et al., 2003; Verhertbruggen et al., 2009) and LM6 (Willats et al., 1998) respectively. We also observed discrete labelling of specific tissues rich in (1 → 4)-β-D-galactan (LM5 binding to border cells), xylogalacturonan (LM8 binding to sloughed off tissue), and xyloglucan (LM15 particularly abundant on root hairs). Examples of these localisations are shown in Fig. 2. LSCM imaging revealed that soil porosity and particle geometry can significantly impact local deposition of polysaccharides. This was clearly demonstrated for xylogalacturonan and (1 → 4)-β-D-galactan (Fig. 2A and Fig. 2B and 2C respectively), both of which are associated with epidermal and border cells. Where roots grew between closely opposed soil particles, ‘pinch points’ were created that appeared to abrade cells from the root surface, thus creating massive local accumulations of cells and their associated glycans (Fig. 2A–2C). We also observed that some cells, cellular material and associated polysaccharide epitopes appeared to become ‘smeared’ onto soil particles, presumably as roots grew through soil pores. An example of this for homogalacturonan is shown in Fig. 2D. These observations are significant because they provide new insight into how interactions between root growth and soil properties contribute to glycan patterning in the rhizosphere, which is likely to in turn impact the local colonisation of microbes with specialised capacity to utilise those glycans.

We further explored associations between root polysaccharides and microbial colonisation by combining both GFP-tagged strains of soil bacteria and mAbs. Bacteria were inoculated onto 0.5 cm² filter paper placed on the top surface of Nafion™ particles in the TSS and allowed to migrate through and freely colonise the TSS (Fig. 3). We visualised GFP-tagged *E. coli* colonies adhering to root hairs (Fig. 3A), and by inoculating with GFP-tagged *Bacillus subtilis* NRS1473 (Hobley et al., 2013) and simultaneously probing with mAb (LM15) that recognises xyloglucan (Marcus et al., 2008), we were able to co-localise *B. subtilis* and xyloglucan on root hairs (Fig. 3B). The observation that xyloglucan is abundant on root hairs and at the elongation zone is consistent with previous work on *in vitro* labelling studies (Freshour et al., 2003; Larson et al., 2014). The co-localisation with bacteria observed in this study may indicate that xyloglucan serves as a nutrient source at this location, thus creating a micro-ecological niche exploitable by microbes with the appropriate CAZymes to utilise xyloglucan.

Our results clearly show that a significant amount of polysaccharide-rich material is retained on or near the root surface, despite the washing steps employed. It seems reasonable to speculate that the same may also be true to roots growing naturally in soils subject to rainwater. Nevertheless, since it is likely that some polysaccharides are washed through the TSS and therefore were not observed, the system only provides partial insight onto glycan rhizodeposits. However, we envisage that the novel methodology described here can make a significant contribution to our understanding of the roles that glycans play in establishing and maintaining rhizobiome diversity. This in turn may help us identify novel means to fine tune rhizobiomes to enhance plant health and crop performance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research was supported by the N8 Agrifood Program Newcastle Pump priming. This work received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Grant agreement No. 647857-SENSOILS).

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